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RAPID INDUCTION OF VIRUS-NEUTRALIZING ANTIBODIES AND VIRAL CLEARANCE IN A SINGLE-SOURCE OUTBREAK OF HEPATITIS C

Jan M. Pestka^{*‡‡}, Mirjam B. Zeisel^{*‡‡}, Edith Bläser^{*}, Peter Schürmann^{*}, Birke Bartosch^{†‡},
Francois-Loïc Cosset^{†‡}, Arvind H. Patel[§], Helga Meisel[¶], Jens Baumert[¶], Sergei Viazov^{**},
Kay Rispeter^{**}, Hubert E. Blum^{*}, Michael Roggendorf^{**}, and Thomas F. Baumert^{*, ††}

^{*}Department of Medicine II, University of Freiburg, Germany, [†]Inserm, U758, Lyon, France,
[‡]Ecole Normale Supérieure de Lyon, Lyon, France, [§]MRC Virology Unit, Institute of
Virology, University of Glasgow, UK, [¶]Institute of Virology, Humboldt-University (Charité),
Berlin, Germany, ^{||}Institute of Epidemiology, GSF National Research Center for Environment
and Health, Neuherberg, Germany, ^{**}Institute of Virology, University of Essen, Germany,
^{††}Inserm, U748, Université Louis Pasteur, Strasbourg, France

^{‡‡}JMP and MBZ contributed equally to this work

Corresponding author: Thomas F. Baumert, M. D., Inserm Unité 748 et Service
d'Hépatogastroentérologie, Université Louis Pasteur, 3 Rue Koeberle, F-67000 Strasbourg,
France; Phone: (+33) 3 90 24 37 02, Fax: (+33) 3 90 24 37 23, e-mail:
Thomas.Baumert@viro-ulp.u-strasbg.fr

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Abbreviations: HCV: hepatitis C virus; HCVpp: HCV pseudoparticles; HVR-1: hypervariable
region-1; MLV: murine leukaemia virus; GFP: green fluorescent protein; EndoH:
endoglycosidase H; mAb: monoclonal antibody.

ABSTRACT

In contrast to a detailed understanding of antiviral cellular immune responses, the impact of neutralizing antibodies for resolution of acute hepatitis C is poorly defined. The analysis of neutralizing responses has been hampered by the fact that patient cohorts as well as hepatitis C virus (HCV) strains are usually heterogeneous and that clinical data from acute-phase and long-term follow-up after infection are not readily available. Using an infectious retroviral HCV pseudo-particle model system, we studied a cohort of women accidentally exposed to the same HCV strain of known sequence. In this single-source outbreak of hepatitis C, viral clearance was associated with a rapid induction of neutralizing antibodies in the early phase of infection. Neutralizing antibodies decreased or disappeared following recovery from HCV infection. In contrast, chronic HCV infection was characterized by absent or low-titer neutralizing antibodies in the early phase of infection and persistence of infection despite the induction of cross-neutralizing antibodies in the late phase of infection. These data suggest that rapid induction of neutralizing antibodies during the early phase of infection may contribute to control of HCV infection. This finding may have important implications for understanding of the pathogenesis of HCV infection and the development of novel preventive and therapeutic antiviral strategies.

INTRODUCTION

Hepatitis C virus (HCV), a member of the *Flaviviridae*, is a major cause of chronic liver disease worldwide (1). The majority of infected individuals develop chronic hepatitis which can then progress to liver cirrhosis and hepatocellular carcinoma. Spontaneous viral clearance occurs in about 20-30% of acutely infected individuals and results in resolution of infection without sequelae.

Both viral and host factors appear to play an important role for resolution of acute infection (2, 3). A large body of evidence suggests, that a strong, multispecific and long-lasting cellular immune response appears to be important for control of viral infection in acute hepatitis C (2-5). In contrast, the impact of neutralizing humoral responses for viral clearance is less defined (3, 6).

Antibodies with HCV neutralizing properties have been first described in experimental infection of chimpanzees (7, 8). These antibodies were directed against epitopes in the hypervariable region-1 (HVR-1) of HCV envelope glycoprotein E2 and appeared to be isolate-specific (7, 8). The presence of antibodies directed against HVR-1 has also been associated with viral clearance in HCV-infected humans (9).

The recent development of novel model systems for the early steps of viral infection including retroviral HCV pseudotypes, HCV-like particles and recombinant infectious virions has finally allowed to detect and characterize neutralizing antibodies in HCV infection (6, 10-15). Nevertheless, the study of the impact of virus neutralizing antibodies for viral clearance in the early phase of infection is hampered by the fact that patient cohorts as well as HCV strains in different patients are very heterogeneous. Furthermore, detailed clinical data from acute-phase and long-term follow-up after infection are not generally available.

Thus, cohorts of patients with a homogenous viral inoculum derived from a single-source, such as the East-German hepatitis C outbreak in 1978/79 involving a large, well-defined cohort of young pregnant women, who received anti-D immunoglobulin contaminated with a single HCV strain (genotype 1b, isolate AD78) (16-18), represent a unique opportunity to
5 study the impact of neutralizing antibodies for control of viral infection.

Retroviral HCV pseudotypes (HCVpp) have been successfully used for the study of viral entry and antibody-mediated neutralization (10-12, 19, 20). We used this model system to evaluate the presence and kinetics of circulating virus-neutralizing antibodies present in the
10 early and late phase in patients in this single-source outbreak of hepatitis C. We demonstrate that in this single-source outbreak of hepatitis C, viral clearance was associated with a rapid induction of neutralizing antibodies in the early phase of infection.

RESULTS

Antibody-mediated neutralization of HCVpp infection derived from strain AD78

isolated from contaminated anti-D immunoglobulin. To study the role of antibody-mediated virus neutralization for viral clearance, we first generated HCVpp bearing E1 and E2 envelope glycoproteins from the consensus sequence of HCV-AD78 isolated from one contaminated batch of anti-D immunoglobulin. HCVpp generated from the established infectious clones HCV-H77C (genotype 1a), HCV-J (genotype 1b) were produced in parallel as controls. Lysates of transfected 293T cells were analysed for HCV envelope protein expression. As shown in Fig. 1A, envelope glycoproteins E1 and E2 derived from isolate HCV-AD78 had molecular masses of ~30-34 kD for E1 and ~60-70 kD for E2 (lane 1-4) similar to envelope glycoproteins derived from HCV-J strain. The different molecular masses of envelope glycoprotein E1 of isolate HCV-AD78 compared to clone HCV-H77C was due to a difference in N-glycosylation, as shown by similar molecular weights of deglycosylated E1 proteins (Fig. 1A, lane 4-8).

As shown in Fig. 1B, HCVpp AD78 efficiently infected Huh7 human hepatoma cells in a dose-dependent and saturable manner, similarly to HCVpp generated from HCV-J strain in parallel (Fig. 1B). Interestingly, the ability of HCVpp AD78 to infect Huh7 cells was 4 times lower than HCVpp derived from HCV infectious clone H77C (Fig. 1B and Fig 4 (supporting information)). For HCVpp AD78 and HCVpp HCV-J (CG1b) saturation of infection was achieved at lower concentrations than for HCVpp derived from HCVpp H77C. Saturation of infection may be due to subviral particles or non assembled envelope proteins present in the HCVpp preparation saturating HCV receptor binding (F.-L. Cosset, unpublished observations). Thus, different ratios of functional HCVpp and non assembled envelope proteins in different isolates may explain the different dose-infection curves for HCVpp derived from different isolates. To identify viral epitopes mediating viral entry of

HCVpp AD78, we tested the ability of several well-defined antibodies directed against HCV envelope glycoproteins to neutralize HCVpp AD78 infection of Huh7 cells. Consistent with our previously published data (21), monoclonal antibody (mAb) AP33, directed against the epitope encompassing amino acid residues 412 to 423 (HCV isolate Gla, genotype 1a), was able to inhibit HCVpp AD78 infectivity up to 97% (Fig. 1C). This inhibition of HCVpp AD78 infection was concentration dependent with an IC_{50} of 0.8 μ g/mL (Fig. 1D). In contrast, mAbs ALP98 (targeting E2₆₄₄₋₆₅₁, HCV Gla-1a) and 2F10 (targeting E2 hypervariable region 1 corresponding to E2₃₉₈₋₄₀₃, HCV isolate BE11, genotype 1b), as well as mAb 49F3 (targeting E2₅₁₆₋₅₃₀, HCV BE11-1b) and 11F11 (targeting E2₄₈₀₋₄₈₇, HCV BE11-1b) had no effect on HCVpp AD78 infectivity (Fig. 1C). These data strongly suggest that epitope E2₄₁₂₋₄₂₃ located within the N-terminal region of envelope glycoprotein E2 plays an important role for HCVpp AD78 entry into Huh7 cells.

Next, we studied whether HCVpp AD78 infection was neutralized by anti-HCV antibodies derived from serum and plasma from HCV-infected individuals. As shown in Fig. 1E, a well characterized anti-HCV positive serum from a patient with chronic hepatitis C inhibiting infection of recombinant infectious HCV particles derived from JFH-1 (13, 14) inhibited HCVpp AD78 entry into Huh7 cells in a dose-dependent manner. In contrast, anti-HCV negative sera from non-infected individuals did not inhibit HCVpp AD78 entry (Fig. 1E).

Taken together, these results demonstrate that antibody-mediated neutralization of infection of Huh7 cells with HCVpp derived from the contaminated anti-D immunoglobulin (AD78) represents a valuable tool to study neutralizing antibody responses in our unique cohort of patients.

Induction of neutralizing antibodies is associated with viral clearance in AD78 single-source outbreak of hepatitis C.

To study the impact of virus-neutralizing antibodies for HCV clearance in women infected by HCV isolate AD78, we studied whether HCVpp AD78 infection of Huh7 cells was inhibited by sera from HCV-AD78 infected individuals. Serum samples from 49 patients that had been infected by HCV isolate AD78 were available to determine neutralizing responses against HCVpp AD78 (including 40 early phase samples and 48 late phase samples). Twenty out of 49 patients resolved infection spontaneously and 29 patients proceeded to chronic hepatitis C without viral clearance. Serial samples were available from 39 patients (including 16 patients and 23 patients with resolved and chronic hepatitis C, respectively).

We first analyzed neutralizing responses in the very early phase of viral infection, corresponding to month 1-6 following the administration of HCV-contaminated anti-D immunoglobulin, to compare resolved versus chronic hepatitis C. Neutralizing responses were quantified by endpoint dilution of sera neutralizing HCVpp AD78 infection of Huh-7 cells. As shown in Fig. 2, a markedly different pattern of neutralizing responses distinguished patients with resolving and chronic HCV infection. Patients with resolved HCV infection (17 patients) were characterized by the rapid induction of neutralizing antibodies with titers reaching up to 1/640 and a median titer of 1/80 in the early phase of infection (range < 1/20 to 1/640). In contrast, patients who subsequently developed persistent HCV infection (23 patients) were characterized by absent or low-titer neutralizing antibodies in the early phase of infection (Fig. 2). In contrast to patients with viral clearance, patients with viral persistence exhibited only a median titer of 1/20 corresponding to a range from < 1/20 to 1/80 in the early phase of infection (Fig. 2). The difference in median titer in the early phase of infection was significant ($p=0.011$).

Analysis of neutralizing responses in late phase of infection revealed a completely different pattern in patients with resolved and chronic HCV infection: in contrast to results obtained during the early phase of infection, only a minority of patients with resolved hepatitis C (19 patients) exhibited antibodies with neutralizing activity 10-17 years following viral clearance.

5 The large majority of patients had lost their neutralizing response against HCV (median neutralizing antibody titer 1/20; range < 1/20 to 1/40). On the other hand, progression of the disease to chronic infection (29 patients) was accompanied by the induction of neutralizing antibodies in the late phase of infection (median titer 1/160, range < 1/20 to 1/640). Despite the small number of patients and samples available for analysis, the differences in antibody
10 responses in patients with resolved and chronic HCV infection were highly statistically significant ($p= 0.001$; Fig. 2). These data indicate that viral clearance is associated with a rapid induction of virus-neutralizing antibodies during the early phase of infection and loss of neutralizing antibodies following viral clearance.

15 We cannot exclude the possibility that neutralizing antibodies were present in antigen-antibody immune complexes and are thus not detected by our in vitro assay. Theoretically, high levels of viremia may bind more neutralizing antibody and unbound neutralizing antibody detected in our assay may predominantly be detected in those patients clearing infection with lower levels of viremia. To address this question we measured HCV RNA
20 levels in individual late phase serum samples from patients with chronic HCV infection (the limited amount of serum did not allow determination of viral load in early phase samples). Since many serum samples with low HCV RNA levels were characterized by the presence of low titer neutralizing antibodies (data not shown), it is unlikely that our assay overestimates the amount of neutralizing antibodies in patients with low HCV RNA levels.

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To investigate the kinetics of neutralizing antibodies in individual patients, we analyzed isolate-specific antibodies in serial serum samples from 16 patients with resolved hepatitis C and 23 patients with chronic infection to compare early versus late phase stratified by disease outcome. Differences of median titer between early and late phase were highly significant for patients with resolved HCV infection ($p=0.002$) and patients who experienced chronic hepatitis C ($p=0.001$). As shown in Fig. 3A, 13 out of 16 patients with resolved hepatitis C exhibited the presence of neutralizing antibodies in the early phase of infection with loss or marked decrease of titer following viral clearance. Two patients were able to eliminate the virus in the absence of any detectable neutralizing antibody response (Fig. 3A, patients 4 and 16) and only one patient exhibited an increase in titer of neutralizing responses following viral clearance (patient 5). In contrast, the majority of patients (14/23) who experienced chronic infection were characterized by absence of neutralizing antibodies or low-titer neutralizing antibodies during the early phase of infection and the emergence of antibodies neutralizing HCV isolate AD78 during the late phase of infection. Interestingly, five patients with chronic infection did not exhibit any detectable neutralizing activity against HCVpp AD78 during both early and late phase of infection (Fig. 3A, patients 17, 18, 27, 30 and 32). The data of neutralizing antibody titers in patients who resolve hepatitis C versus patients who develop chronic hepatitis C is meaningful only if the sampling times during the acute phase are comparable in the two groups. To address this question, time of sampling was plotted versus neutralizing antibody titer (Fig. 3B). As shown in Fig. 3B, sampling times (measured in days post infection) were comparable in both groups ($p = 0.91$; student's t-test). Furthermore, there was no significant correlation between sampling time and neutralizing titer as assessed by Spearman correlation coefficient ($p>0.5$). Similar results were obtained for early phase samples presented in Fig. 2 (data not shown). These findings clearly rule out sampling times as a confounding factor for the observed differences in neutralizing responses.

Cross-neutralization of heterologous HCV strains. Next, we assessed the ability of antibodies neutralizing AD78 to cross-neutralize heterologous HCV strains from a different sub- or genotype. Similar to previous reports (12, 13), antibodies neutralizing the inoculating virus in the chronic phase of infection were able to cross-neutralize infection of HCVpp
5 derived from heterologous strains including HCV-J (1b), H77C (1a) and UKN2A.2.4 (2) (Fig. 5 supporting information). All patients with chronic hepatitis C cross-neutralized at least one heterologous strain and 21 out of 23 patients (91%) with chronic hepatitis C were able to cross-neutralize at least 2 heterologous strains in the late phase of infection (data not shown). Interestingly, all individuals with non detectable HCV-AD78 neutralizing antibodies were
10 able to efficiently neutralize HCVpp derived from at least one other sub- or genotype (Fig. 5 supporting information and data not shown).

Finally, we addressed the question whether the degree of heterologous neutralizing activity in the early phase of infection was related to outcome of infection. To address this
15 question, we studied cross-neutralizing activity of sera from the early phase of infection of patients presented in Fig. 2. Interestingly, 13 out of 17 patients (76%) with resolved hepatitis C but only 10 out of 23 patients (43%) progressing to chronic hepatitis C cross-neutralized at least one heterologous strain in the early phase of infection ($p=0.037$; Fig. 6 supporting information). Although the number of patients is small, these data may suggest that patients
20 with resolution of infection may exhibit a broader cross-neutralizing activity of antiviral antibodies in the early phase of infection.

DISCUSSION

Studying antibody-mediated virus neutralization in a single-source outbreak of HCV infection with the identical viral inoculum received by all patients, we demonstrate that viral clearance is associated with the rapid induction of neutralizing antibodies in the acute phase of infection and loss of neutralizing antibodies following recovery from infection. In contrast, chronic HCV infection is characterized by a complete absence or reduced capacity to neutralize the transmitted virus in the early phase of infection and a delayed induction of neutralizing antibodies in the late phase of infection.

Until now, studies of the neutralizing potential of antibodies were limited by the fact that the viral inoculum is poorly defined, thus precluding the detection of isolate-specific antibodies. In this study, serum samples collected early after infection and for over 17 years after a single-source outbreak of HCV, enabled us for the first time to study the role of isolate-specific neutralizing antibodies for HCV clearance in humans. This East-German anti-D cohort is unique by the fact that it meets several criteria required to constitute an ideal study to assess the natural history and pathogenesis of HCV infection since it includes a precisely defined onset of infection from a single infected source and a prospective, comprehensive follow-up of long duration (22).

Previous studies assessing antibody-mediated neutralization in acute hepatitis C failed to reveal a clear association between neutralizing antibodies and viral clearance (11, 13). In contrast, our study demonstrates a significant association between the rapid induction of virus-neutralizing antibodies in the early phase of infection and viral clearance. The main difference between previous studies and this study is the level of heterogeneity in viral isolates and recipients. Whereas previous studies assessed antibody-mediated virus neutralization in patient cohorts infected with different viral isolates, our cohort was

characterized by a single-source inoculum allowing us to match the envelope of the model virion with infectious particles of the AD78 isolate received by the individual patients. The generation of autologous HCVpp of isolate AD78 enabled us to assess strain-specific neutralizing activity in the individual patient and correlate the quality and quantity of this activity with outcome. Thus, this approach eliminates viral-specific factors in the analysis of virus neutralization such as mismatch between envelope glycoproteins of the model viral particle and viral isolate of the patient. The question of viral isolate specific factors was also addressed in a from Lavillette *et al.* who studied nosocomial acquired HCV infection in hemodialysis patients (12). In this study of patients co-infected with two different viral isolates, Lavillette and colleagues demonstrated evidence that viral load appears to correlate with the presence of neutralizing humoral antibodies during the acute phase of infection. Since this patient cohort of hospitalized patients was quite heterogeneous the impact of other factors contributing to control of HCV infection was difficult to assess. In contrast, the East-German HCV single-source cohort comprises a very well defined homogenous group of young healthy women thus largely excluding host factors related to sex, age and co-morbidity.

The characteristics of patients available to analysis in this study were similar to the characteristics of patients of the entire cohort (23) as shown by age (median age at infection 24 years, range 18-37 years), sex (all female), mode of transmission (all IV), viral inoculum (all HCV-AD78), outcome of viral infection (41%) and the absence of co-morbidity. Thus, this finding suggests that the results obtained in this study are representative for mechanisms of viral clearance of the cohort in general. Although we cannot exclude that patients who develop chronic HCV infection exhibited an underlying subtle deficiency in immune responsiveness to HCV that results in a failure to mount the immune response that accounts

for clearance, long-term evaluation of patients did not reveal the presence of a clinically evident innate or acquired immunodeficiency (23).

Interestingly, we observed a loss or decline of neutralizing antibodies in patients with
5 successful viral clearance (Fig. 2-3). This finding may have important implications for the understanding of immune responses protecting against HCV re-infection. Loss of neutralizing antiviral immune responses as observed in this study may contribute to an absent or limited protective immunity against re-infection as observed in humans (24, 25).

10 Progression to chronic HCV infection was characterized by a delayed induction of neutralizing antibodies in the late phase of infection (Fig. 3). These results suggest that HCV can persist despite the induction of antibodies neutralizing the transmitted virus. Viral escape from antibody-mediated neutralization in these individuals may occur on several levels. First, HCV exists as a quasispecies with distinct viral variants in infected individuals changing
15 constantly over time. This variability has been shown to represent a mechanism of escape from antibody-mediated neutralization in the chimpanzee model (8). Second, the interplay of HCV glycoproteins with high-density lipoprotein and the scavenger receptor BI has been shown to mediate protection from neutralizing antibodies present in sera of acute and chronic HCV-infected patients (26, 27). Third, as shown for other viruses such as human
20 immunodeficiency virus, escape from neutralizing antibodies may occur through a combination of different mechanisms, for instance point mutations, insertions/deletions or changes in glycosylation patterns of the viral envelope (28) or conformational masking of receptor binding sites following envelope-antibody interaction (29) preventing neutralizing antibody binding. In this respect, it is of interest to note that viral variants of transmitted virus
25 HCV-AD78 have been shown to emerge rapidly in patients progressing to chronic HCV infection (30). Forth, an impaired ability to cross-neutralize these variants may contribute to

viral escape. This hypothesis is supported by our observation that cross-neutralizing activity of antibodies in the acute phase of patients with resolved hepatitis appeared to be more efficient than in patients with progression to chronic disease (Fig. 6 supporting information).

5 Interestingly, we observed that several patients (#17, 18, 32, 34) with chronic HCV infection were not able to mount an AD78-specific neutralizing response, although the induced antiviral antibodies were able to neutralize other HCV strains (Fig. 5, supporting information). Further studies are under way to assess whether the inability to induce AD78-specific antibodies in these patients may be due to viral quasispecies evolution with loss of
10 AD78 epitopes.

A previous study assessing cellular immune responses in the same cohort has demonstrated that patients who resolved HCV infection were characterized by a strong and multispecific antiviral cellular immune response 18-20 years following viral clearance
15 suggesting that the induction of HCV-specific cellular immune responses play an important role for viral clearance (18). The association of a rapid emergence of neutralizing antibodies with resolving infection in this cohort suggests that better early neutralizing responses assist in clearing HCV replication. The absence of replication in patients who clear the virus results in the lack of antigen boosting to high levels of late phase antibody, while chronically infected
20 patients continue to generate antibody responses.

On the other hand, we can not exclude that clearance may be exclusively mediated by cellular responses. Failure to clear HCV replication may result in very high levels of antigen production which act as a sink for newly generated antibody. Continuing replication boosts
25 high levels of antibody production - this replication permits continuing neutralization escape by the currently successfully replicating HCV.

5 However, experimental data obtained in animal models have demonstrated that immune control of poorly cytopathic viruses, such as lymphocytic choriomeningitis virus (LCMV) or simian immunodeficiency virus requires a collaboration of both the cellular and humoral arms of the immune system (31, 32). Indeed, gene-targeted mice expressing the immunoglobulin heavy chain of virus-neutralizing antibodies exhibit an accelerated LCMV clearance (33). Applying these findings to HCV infection - another prototype of persistent-prone non-cytopathic viruses - it is highly probable, that clearance of HCV is mediated by an orchestrated interplay of CTL (18) and neutralizing responses (our study). This conclusion is further supported by recent findings for HIV demonstrating that neutralizing antibodies act in concert with antiviral cellular responses for control of HIV infection (28, 34, 35).

10 Furthermore, a causal role of neutralizing responses for control of HCV infection seems highly probable when applying the widely accepted Bradford Hill criteria assessing the probability of causality of an association (36): The relationship between early neutralizing responses and viral clearance is remarkably strong (Fig. 2, 3). The time course of neutralizing antibodies in relationship to viral clearance in the acute phase of self-limited hepatitis C indicates temporality of the relationship (Fig. 2, 3). A high titer of neutralizing antibody in the early phase correlated with outcome suggesting a dose-dependency of the relationship (all patients exhibiting an anti-AD78 titer of $\geq 1:160$ in the early phase cleared infection). Other variables assessed in this study (age, ALT levels) did not predict outcome (data not shown) suggesting specificity. Similar evidence for control of HCV infection in a different cohort (12) suggests consistency of the observation. Finally, as discussed above, experimental studies in animal models (31-33) and clinical studies in humans ((28, 34, 35) demonstrate coherency and analogy of results with previous knowledge obtained for other viruses.

Finally, our findings may not only have important implications for the understanding of the pathogenesis of HCV infection, but also for the design of novel immunotherapeutic and preventive vaccine approaches.

MATERIALS AND METHODS

Patients. Between August 1978 and March 1979, 14 batches of the same virus-contaminated anti-D immunoglobulin had been administered to 2867 women in East-Germany for prevention of haemolytic disease in newborns through Rhesus incompatibility, leading to a single-source outbreak of HCV infection (16). The HCV isolate obtained from one of the batches of the contaminated globulin was designated HCV-AD78 (genotype 1b) (17). A total of 88 serum samples from 49 patients of this outbreak were available to study the antibody response to HCV-AD78. Serum samples available for this study had been drawn during the early phase of infection (1 to 6 months after infection) as well as 10 to 17 years after infection. Resolved infections were characterized by serum HCV RNA levels that became undetectable during the first 6 months after infection whereas sera from chronic infections tested positive for HCV RNA as long as after 10 to 17 years after infection. None of the patients had received interferon-based antiviral therapies prior to sampling of the late phase samples.

Antibodies and cell lines. Horseradish peroxidase-conjugated goat anti-mouse antibodies were from Amersham Biosciences (Piscataway, NJ). Mouse monoclonal anti-E2 (3E5-1, AP33, ALP98 and 2F10), anti-E1 (11B7) and anti-galactosidase (anti-LacZ) antibodies have been described (37, 38). Chimpanzee anti-E2 49F3 and 11F11 (directed against amino acid residus 516 to 530 and 480 to 487 of the HCV polyprotein, respectively) as well as 2F10 were kindly provided by E. Depla (Innogenetics) (13, 37, 39, 40). Huh7 and 293T cells were maintained as described (19, 37, 41-43).

Antibody-mediated inhibition of HCVpp infection. HCVpp derived from strain AD78 (17) were generated as described in supporting information. Control pseudoparticles (19) without envelope glycoproteins (control pp) and HCVpp derived from strains H77C, HCV-J (CG1b)

and UKN2A.2.4 were generated as described previously (12). For analysis of HCV protein expression, 293T cells were washed, lysed and subjected to SDS-polyacrylamide gel electrophoresis followed by immunoblotting as described (36). For the study of antibody-mediated neutralization, Huh7 cells were seeded the day before infection assays in 48-well plates at a density of 0.5×10^4 cells/well. HCVpp were mixed with anti-HCV or control serum (starting at a dilution of 1/20) and pre-incubated for 1 h at room temperature. This mixture was then added to Huh7 cells and incubated for 3 h at 37°C. The supernatants were removed and cells incubated in regular medium for 72 h at 37°C and HCV entry was determined by analysis of GFP reporter gene expression as described (19). Antibody mediated neutralization was assessed by the specific infectivity of HCVpp in the presence or absence of serum as described in supporting information.

Statistical analysis. Differences of titer concentrations between resolved and chronic hepatitis C were tested by the Mann-Whitney U test. The Wilcoxon signed rank test for paired data was used to assess differences of titer concentrations in early and late phase. Associations of titer concentration in dichotomous form (0: < cut-off value, 1: \geq cut-off value) with phase status were tested by McNemar's test (for paired data). Correlation of sampling time and titers were assessed by Spearman correlation coefficient. For all statistical analyses a P value less than 0.05 was considered to be statistically significant. All evaluations were performed with the statistical software package SAS (Version 9.1 for Windows, SAS-Institute Inc., Cary, NC, USA).

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CONTRIBUTORS

T. F. B., M. R., H. M. and M. B. Z. designed research; J. M. P., M. B. Z., E. B., P. S. and S. V. performed research; F.-L. C., B. B., A. H. P., H. M., K. R., M. R. contributed new reagents/analytical tools; J. M. P., M. B. Z., E. B., H. M., J. B., M. R. and T. F. B. analyzed
5 data; T. F. B., M. B. Z., H. E. B., and M. R. wrote the paper. J. M. P. and M. B. Z. contributed equally to this work.

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FIGURE LEGENDS

Fig. 1. Synthesis and characterization of HCVpp derived from HCV isolate AD78. (A) HCV protein expression in 293T-transfected cells. 293T cells were transfected as described in Materials and Methods including an expression vector coding for HCV envelope glycoproteins of isolate AD78 from the contaminated anti-D immunoglobulin batches. 293T-transfected cells, expressing HCVpp H77C, HCVpp AD78, HCVpp J or control pp were lysed and proteins were incubated in the presence or absence of endoglycosidase H (EndoH) and separated by SDS-PAGE, followed by immunoblotting with anti-E1 mAb (11B7) and anti-E2 mAb (3E5). The positions of the molecular weight markers in kilodalton (kD) are shown. (B) Infectivity of HCVpp. Huh7 cells were incubated with HCVpp for 3 h at 37°C. HCVpp H77C (■), HCVpp AD78 (◇), HCVpp J (▲) and control pp (□) entry into Huh7 cells was determined by measuring expression of GFP reporter gene by flow cytometry after 72 h. (C) Inhibition of HCVpp AD78 by anti-E2 antibodies. Antibodies (50 µg/mL) were preincubated with HCVpp AD78 for 1 h at room temperature. This mixture was then added to Huh7 cells and incubated for 3 h at 37°C. HCVpp entry into Huh7 cells was determined as described above. (D) Dose-dependent inhibition of HCVpp AD78 entry by mAb AP33. HCVpp AD78 were preincubated with different concentrations of mAb AP33 (▲), ALP 98 (◇) or control IgG (■) for 1 h. This mixture was then used to infect Huh7 cells as described above. (E) Dose-dependent inhibition of HCVpp AD78 entry by an anti-HCV positive serum from a patient with chronic hepatitis C. HCVpp AD78 were preincubated for 1 h with different dilutions of sera from a patient with chronic HCV (■) or from a anti-HCV negative individual (◇). This mixture was then used to infect Huh7 cells as described above. Data are expressed as means of triplicate determinations ± SD from one out of at least three independent experiments. HCVpp – HCV pseudoparticles containing HCV envelope glycoproteins; control pp - control pseudoparticles without envelope glycoproteins; E1+ and E2+ - deglycosylated forms of E1 and E2.

Fig. 2. Neutralizing antibodies in patients with resolved or chronic hepatitis C. Anti-HCVpp neutralizing titers were determined by end-point dilution of sera. HCVpp AD78 or control pp core were pre-incubated for 1 h with serial serum dilutions before infection of Huh7 target cells. The end-point titers of the early (1-6 months post-infection) and late phase (10-17 years post-infection) serum samples are shown as scatter plots. The median titer is marked by a line. Data are expressed as means of two independent experiments performed in duplicate. Samples showing a titer of less than 1/20 were considered negative. The cut-off titer 1/20 is indicated by a dotted line.

Fig. 3. Neutralizing antibodies in serial serum samples of individual patients. (A) Kinetic of neutralizing antibodies in serial serum samples of individual patients. Titers of anti-HCVpp neutralizing antibodies were determined by end-point dilution of serial serum samples from 16 patients with resolved and 23 patients with chronic hepatitis C. Patients' identification numbers are indicated in boxes. Titers of neutralizing antibodies present in the early and late phase of infection in individual patients are connected through lines. (B) Plotting of sampling time (in days post infection) versus neutralizing antibody titer during the acute phase of infection. Samples showing a titer of less than 1/20 were considered negative. The cut-off titer 1/20 is indicated by a dotted line.